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Induction of CRE-Mediated Gene Expression by Stimuli That Generate Long-Lasting LTP in Area CA1 of the Hippocampus

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Summary

Gene expression regulated by the cAMP response element (CRE) has been implicated in synaptic plasticity and long-term memory. It has been proposed that CRE-mediated gene expression is stimulated by signals that induce long-term potentiation (LTP). To test this hypothesis, we made mice transgenic for a CRE-regulated reporter construct. We focused on long-lasting long-term potentiation (L-LTP), because it depends on cAMP-dependent protein kinase activity (PKA) and de novo gene expression. CRE-mediated gene expression was markedly increased after L-LTP, but not after decremental LTP (D-LTP). Furthermore, inhibitors of PKA blocked L-LTP and associated increases in CRE-mediated gene expression. These data demonstrate that the signaling required for the generation of L-LTP but not D-LTP is sufficient to stimulate CRE-mediated transcription in the hippocampus.

Introduction

Learning has at least two functionally and mechanistically distinct components: a short-term phase that lasts no more than several hours, and a long-term component that can continue for days or longer (Barondes and Cohen, 1968; Kesner and Connor, 1972; Goelet et al., 1986; Squire and Morgan, 1988; Alvarez et al., 1994; Cahill et al., 1994). In invertebrate and vertebrate learning models, the formation of long-term memory requires de novo RNA transcription and protein synthesis (Flexner and Flexner, 1966; Squire and Barondes, 1973; Grechsch and Matthies et al., 1980; Montarolo et al., 1986; Castellucci et al., 1989; Crow and Forrester, 1992; Tully et al., 1994). For example, associative conditioning in Aplysia and Drosophila requires new gene expression during a critical period following training. Another common feature of long-term memory in vertebrates and invertebrates is a dependency on the cAMP and Ca^{2+} signal transduction systems (Livingston et al., 1984; Yovell et al., 1987; Feany, 1990; Abrams et al., 1991; Levin et al., 1992; Silva et al., 1992; Frank and Greenberg, 1994; Wu et al., 1995). For example, Ca^{2+}/calmodulin-sensitive adenylyl cyclases may play a role in associative learning in Aplysia, associative olfactory learning in Drosophila, and some forms of spatial learning in mice (Livingston et al., 1984; Yovell et al., 1987; Feany, 1990; Abrams et al., 1991; Wu et al., 1995).

LTP is an activity-dependent strengthening of synaptic efficacy that can last for days or weeks in intact animals, and may be a useful model for studying learning-induced changes in synaptic efficacy (Bliss and Lomo, 1973; Teyler and Discenna, 1984; Bear and Malenka, 1994; Collingridge and Bliss, 1995). In an interesting parallel to learning and memory, LTP has decremental (1–3 hr) and long-lasting forms (>3 hr) in the hippocampus (Matthies et al., 1990; Collingridge and Bliss, 1995). D-LTP can be generated by a single high frequency stimulus, and is generally believed to be mediated via covalent modification of proteins by kinases or phosphatases. L-LTP, which is generally produced by multiple trains of high frequency stimulation, can last hours or even days, and is sensitive to inhibitors of transcription and translation (Krug et al., 1984; Frey et al., 1988; Matthies et al., 1990; Nguyen et al., 1994). Inhibitors of cAMP-dependent protein kinase (PKA) attenuate both the early and late components of L-LTP but not D-LTP (Matthies and Reymann, 1993; Frey et al., 1993; Huang and Kandel, 1994; Blitzer et al., 1995). Furthermore, activators of PKA and adenylyl cyclase applied extracellularly generate L-LTP in area CA1 of the hippocampus (Frey et al., 1993; Huang and Kandel, 1995; Slack and Walsh, 1995). Collectively, these observations suggest that cAMP-mediated transcription may be important for L-LTP.

The cAMP response element (CRE) is a *cis*-acting enhancer that mediates cAMP and Ca^{2+}-stimulated transcription via CREB and the closely related ATF1 and CREMτ transcription factors (Silver et al., 1987; Deutsch et al., 1987; Montminy and Bilezikjian, 1987; Hoeffler et al., 1988; Van-Nguyen et al., 1990; Sheng et al., 1990; de Groot et al., 1993). CREB integrates increases in cAMP and Ca^{2+} by inducing synergistic increases in CRE-mediated transcription (Montminy et al., 1990; Van-Nguyen et al., 1990; Sheng et al., 1990; Kilbourne et al., 1992; Impey et al., 1994). This led to the proposal that the CREB family of transcription factors could serve to couple temporally overlapping neuronal signals to long-term changes in synaptic strength.

Long-term facilitation of sensory neurons in Aplysia requires CRE-mediated gene expression (Kaang et al., 1993; Dash et al., 1990). Furthermore, relief of repression by the inhibitory CREB2 isoform converted transient facilitation in Aplysia into long-term facilitation (Bartsch et al., 1995). In Drosophila, expression of an inhibitory CREB or ATF homolog-blocked long-term memory (Yin et al., 1994), whereas expression of an activating isoform of CREB greatly enhanced the acquisition of long-term memory (Yin et al., 1995). In addition, mutant mice lacking CREB isoforms α and δ have deficiencies in the long-term retention of several learning tasks and markedly attenuated LTP (Bourtchuladze et al., 1995). These results suggest that CRE-regulated transcription may be important for learning and LTP. However, it is difficult to determine whether a phenotype, caused by a loss of function, is the result of a direct mechanistic defect. For example, the aberrant LTP in CREB knockout mice could

be the result of a preexisting alteration of synaptic function rather than the loss of CREB-dependent gene expression immediately following LTP induction or learning. It is not known whether LTP or any other form of vertebrate synaptic plasticity actually causes an increase in CRE-directed gene expression. Therefore, we made mice transgenic for a CRE-β-galactosidase reporter construct (CRE-LacZ) to test the hypothesis that stimuli that generate L-LTP increase CRE-mediated gene expression.

Results

Regulation of CRE-Regulated Gene Expression in Cultured Neurons

To determine if CRE-LacZ transgenic mice had regulated expression of β-galactosidase, cultures of primary neurons from transgenic mouse founder lines were treated with agonists that increase cAMP, and assayed for β-galactosidase. Forskolin and SKF-38393, a dopamine D1/D5 agonist, stimulated β-galactosidase expression in 3 of 8 founder lines (Figure 1A). We also examined the induction of CRE-LacZ expression in cultured hippocampal neurons by immunocytochemistry (Figures 1B–1E). Glutamate induced CRE-LacZ expression in the presence (Figure 1D) but not in the absence of extracellular Ca^{2+} (data not shown). In agreement with an earlier study (Bading et al., 1993), we found that NMDA did not generate a detectable increase in CRE-driven LacZ expression in cultured hippocampal neurons (Figure 1E).

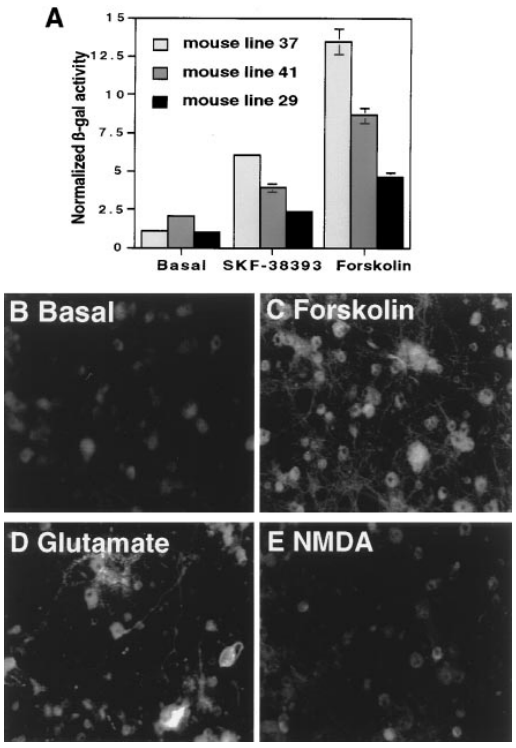

Figure 1. Induction of CRE-Mediated Gene Expression in Cultured Neurons and Hippocampal Slices from CRE-LacZ Mice by Agonists that Increase cAMP

(A) Forskolin (50 μM) and SKF-38393 (20 μM) stimulation of β-galactosidase expression in cultured hippocampal neurons from three CRE-LacZ mouse lines. Error bars represent standard deviation of duplicate determinations. CRE-LacZ mice from line 37 were used in subsequent experiments. (B) Immunocytochemistry for β-galactosidase was performed on hippocampal cultures treated with vehicle, 50 μM forskolin (C), 25 μM glutamate (D), and 25 μM NMDA (E). Confocal images are at 600 × (original) magnification.

L-LTP but Not D-LTP Stimulated CRE-Mediated Gene Expression

The best characterized form of transcriptionally dependent synaptic plasticity is L-LTP of the Schaffer collateral-CA1 pyramidal cell synapse. We differentiated D-LTP from L-LTP in area CA1 by varying the number of tetani (Figure 2A). L-LTP was consistently produced with three 100 Hz, one s tetani at 5 min intervals, and D-LTP was generated with a single, 100 Hz, one s tetanus. The D-LTP stimulus paradigm did not significantly increase CRE-mediated gene expression relative to low frequency stimulus (LFS) controls (Figure 3B). Tetani that produced L-LTP markedly increased β-galactosidase expression in the pramidal cell layer and in a few scattered cells in the stratum oriens (Figures 3B and 3E). Increased β-galactosidase expression was detectable two hr posttetanus, and reached a maximum after 4–6 hr (data not shown). It is interesting that RNA transcription-dependent L-LTP has a similar time-course (Frey et al., 1988; Nguyen et al., 1994). These data show that tetani that generated L-LTP increased CRE-mediated gene expression, and are consistent with a causal role for CRE-mediated gene expression in L-LTP.

Regulation of L-LTP Associated Increases in CRE-LacZ Expression by Protein Kinases

Activation of PKA is necessary and sufficient for L-LTP but not D-LTP in the rat hippocampus (Matthies and Reymann, 1993; Frey et al., 1993; Blitzer et al., 1995; Slack and Walsh, 1995). Furthermore, L-LTP generated by direct activation of PKA requires de novo RNA transcription and gene expression (Nguyen et al., 1994). In agreement with previous work, treatment of slices with the adenylyl cyclase activator, forskolin, evoked L-LTP in area CA1 of the hippocampus (Figure 3A). Forskolin and the D1/D5 agonist SKF-38393 (data not shown) also increased β-galactosidase expression in neurons throughout area CA1 (Figures 3C and 3E). The inactive forskolin homolog, 1,9-dideoxy-forskolin, did not generate LTP or a significant increase in β-galactosidase expression relative to untreated controls ($p>0.5$, controls $n = 5$, 1,9-dideoxy-forskolin $n = 5$). Furthermore, tetanization using the paradigm that generated L-LTP occluded further increases in fEPSP slope, or normalized CRE-LacZ expression by forskolin (Figures 3B and 3C). These data suggest that activation of adenylyl cyclase and PKA are sufficient for the increases in CRE-mediated gene expression associated with L-LTP.

We also measured the effect of the PKA inhibitors, H89 and KT5720, on L-LTP and CRE-mediated gene

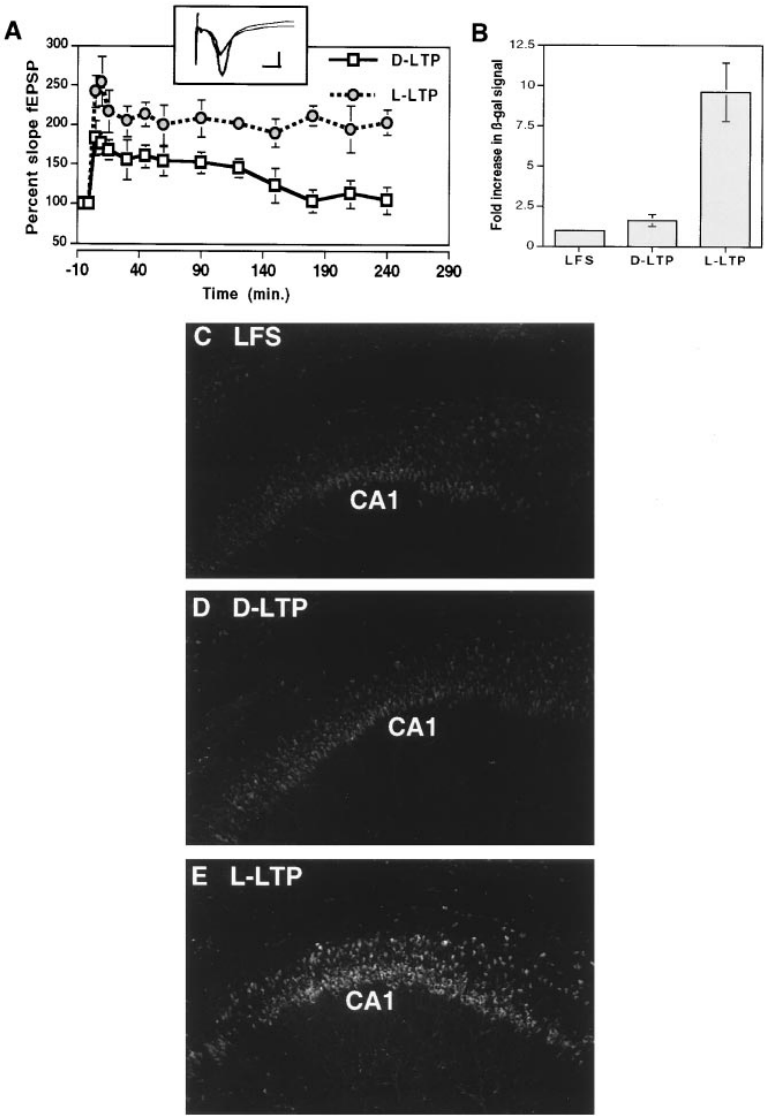

Figure 2. Increased CRE-Mediated Gene Expression Associated with L-LTP but Not D-LTP

(A) L-LTP (closed circles) was produced with three 100 Hz, one s tetani at 5 min intervals, and D-LTP (open squares) was generated by a single 100 Hz, one s tetanus. Inset depicts representative EPSPs from a L-LTP trace before and after potentiation (Calibration: 5 ms, 0.3 mV).

(B) A bar graph depicting the fold increase in β-galactosidase immunocytochemistry for slices that generated D-LTP ($p > 0.2$, D-LTP $n = 10$, LFS $n = 21$) and L-LTP ($p < 0.001$, LFS $n = 21$, L-LTP $n = 13$) relative to LFS control slices. Slices were fixed 4 hr post-tetanus.

(C–E) Representative examples of immunocytochemistry for β-galactosidase expression associated with low frequency stimulation (LFS) (C), D-LTP (D), or L-LTP (E). Images are at 200 × (original) magnification. Error bars show SEM.

expression. H89 attenuated L-LTP in area CA1 (Figure 4A) and inhibited increases in CRE-LacZ expression associated with L-LTP (Figures 4B and 4D). The selective PKA inhibitor, KT-5720, also attenuated L-LTP (Figure 4A) and increases in CRE-regulated gene expression (Figures 4B, 4E, and 4F). These data indicate that both L-LTP and the increases in CRE-mediated gene expression associated with L-LTP require PKA activity.

Ca^{2+}/calmodulin-regulated kinases (CaMKs) have been implicated in LTP and in the regulation of CRE-mediated gene expression (Ito et al., 1991; Silva et al., 1992; Enslen et al., 1994; Sun et al., 1994; Matthews et al., 1994). We found that the CaMK inhibitor, KN-62, prevented both LTP and the associated increases in CRE-LacZ expression (data not shown). Because KN-62 impaired paired pulse facilitation, a measure of presynaptic function, mechanistic interpretation of these results is difficult. Nevertheless, the finding that both LTP and LTP-associated increases in CRE-LacZ expression were blocked by KN-62 further supports a causal relationship between the two.

L-LTP and Associated Increases in CRE-Mediated Gene Expression Require L-Type Calcium Channel Activation but Not NMDA Receptor Function

Although D-LTP in area CA1 requires NMDA receptor activation, several labs have reported CA1 LTP that does not require NMDA receptor activation (Grover and Teyler, 1990; Aniksztejn and Ben Ari, 1991; Huang and Malenka, 1993; Grover and Teyler, 1994; Hanse and Gustafsson, 1995). Furthermore, NMDA-independent long-term potentiation in area CA1 persists for greater than 4 hr (Grover and Teyler, 1994). On the other hand, it should be noted that in rat hippocampal slices, NMDA inhibitors markedly reduced L-LTP (Reymann et al., 1989). In mice, we found that the NMDA receptor in-

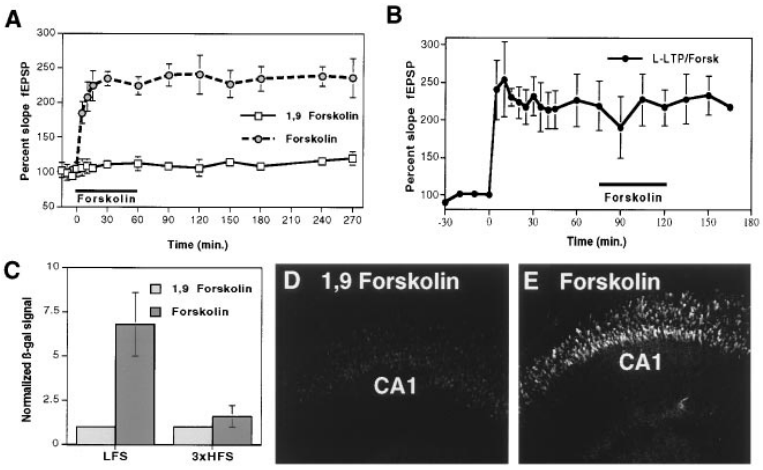

Figure 3. Activation of Adenylyl Cyclase and PKA Generates L-LTP and Increases CRE-LacZ Expression

(A) Treatment of hippocampal slices with 50 μM forskolin (circles), but not 50 μM 1,9-dideoxy-forskolin (squares) produces lasting potentiation of the Schaffer collateral synapse. Slices were fixed 5 hr after forskolin treatment.

(B) Pre-established L-LTP occludes further potentiation by forskolin (50 μM). Slices were fixed 5 hr after tetanization.

(C) Fold increase in β-galactosidase immunocytochemistry generated by forskolin in untetanized hippocampal slices (LFS) versus slices stimulated by the triple tetanus used to generate L-LTP. The data was normalized so that 1,9 dideoxy-forskolin treatments are equal to one.

(D–E) Representative examples of immunocytochemistry for β-galactosidase performed on 50 μM 1,9-dideoxy-forskolin treated hippocampal slices (D) or slices treated with 50 μM forskolin (E). Images are at 200 × (original) magnification. Error bars show SEM.

hibitor APV completely blocked D-LTP but that the combination of APV and MK801 only moderately reduced L-LTP in area CA1 of the hippocampus (Figure 5A). This apparent discrepancy between our results and the earlier observations in rat could be caused by species differences or methodological differences. Nevertheless, our results suggests that L-LTP may require another source of Ca^{2+} influx, such as the activation of voltage-gated Ca^{2+} channels by AMPA/kainate receptor-mediated depolarization. Although the inhibition of NMDA receptor function markedly reduced L-LTP, we found that there is a component of L-LTP that appears to be independent of NMDA receptor function (Figure 5A). Interestingly, APV did not prevent increases in CRE-mediated gene expression associated with L-LTP (Figures 5B and 5D). Furthermore, this experiment indicates that the stimulation of CRE-mediated gene expression associated with L-LTP occurs independently of D-LTP.

The generation of L-LTP even in the presence of NMDA inhibitors may be due to a sufficiently large influx

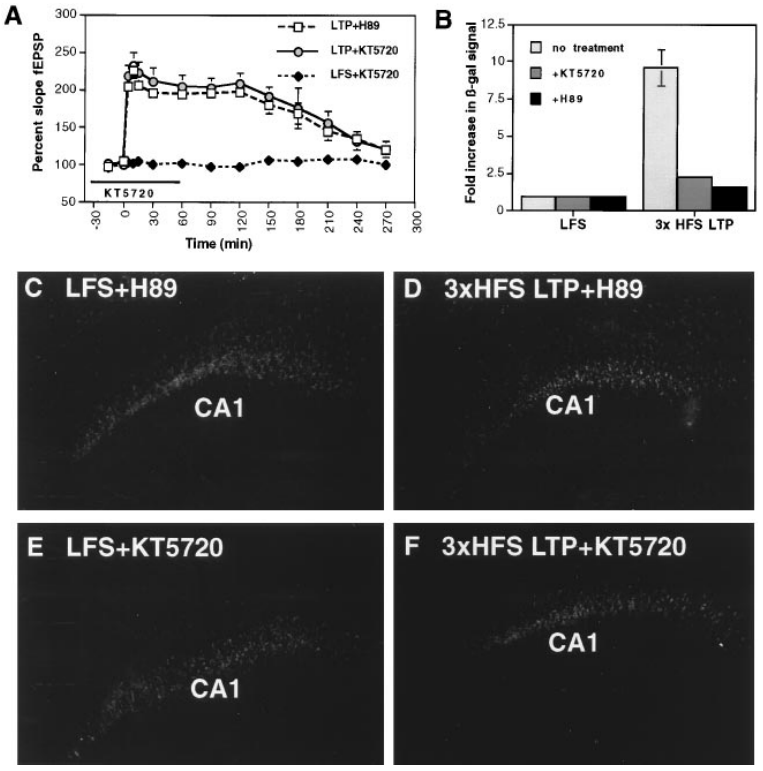

Figure 4. Inhibition of L-LTP and Associated Increases in CRE-Mediated Gene Expression by PKA Inhibitors

(A) Tetani that normally produce L-LTP did not generate persistent LTP in the presence of 10 μM H89 (squares) or 250 nM KT5720 (circles).

(B) Fold increase in β-galactosidase immunocytochemistry after three 100 Hz, one s tetani (3 × HFS) in the presence and absence of the PKA inhibitors H89 ($p < 0.02$, LFS $n = 10$, LTP $n = 7$) and KT5720 ($p < 0.05$, LFS $n = 7$, LTP $n = 6$). Error bars show SEM. Slices were fixed 5 hr posttetanus.

(C–D) Representative examples of CRE-LacZ expression in a H89-treated LFS control (C) and after three 100 Hz, one s tetani at 5 min intervals in the presence of H89 (D).

(E) Representative examples of CRE-LacZ expression in a KT5720-treated LFS control and (F) after three 100 Hz, one s tetani at 5 min intervals in the presence of KT5720. Images are at 200 × (original) magnification.

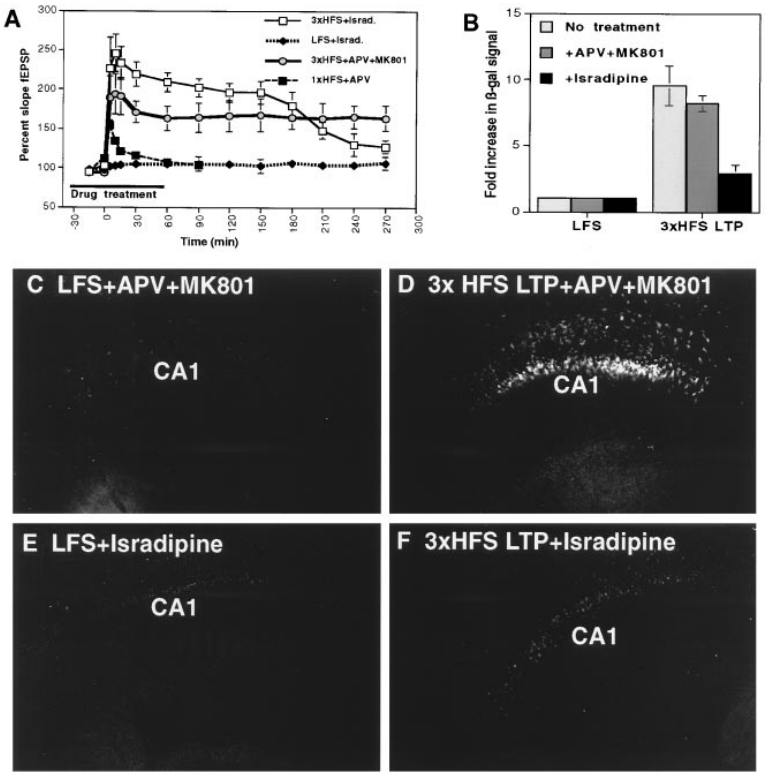

Figure 5. L-LTP and Associated Increases in CRE-LacZ Expression Require L-type Ca^{2+} Channel Activity but Not NMDA Receptor Activity

(A) L-LTP in area CA1 was unaffected by the combination of 50 μM APV and 20 μM MK801 (circles) while the L-type Ca^{2+} channel blocker isradipine (5 μM) attenuated L-LTP (open squares). D-LTP was blocked by 50 μM APV (closed squares).

(B) Fold increase in β-galactosidase immunocytochemistry after three 100 Hz, one s tetani (3 × HFS) in the presence and absence of APV and MK801 ($p < 0.01$, LFS $n = 6$, LTP $n = 5$) or isradipine ($p < 0.05$, LFS $n = 7$, LTP $n = 6$). Slices were fixed 4 hr posttetanus.

(C–D) Representative examples of CRE-LacZ expression in a APV+MK801-treated LFS control (C) and after three 100 Hz, one s tetani at 5 min intervals in the presence of APV+MK801 (D).

(E–F) Representative examples of CRE-LacZ expression in an isradipine treated LFS control (E) and after three 100 Hz, one s tetani at 5 min intervals in the presence of isradipine (F). Images are at 200 × (original) magnification. Error bars show SEM.

of Ca^{2+} through voltage gated Ca^{2+} channels (VGCCs). Several studies have shown that NMDA receptor-independent LTP at the Schaffer collateral-CA1 pyramidal cell synapse depends on the activation of VGCCs (Grover and Teyler, 1990; Aniksztejn and Ben Ari, 1991; Hanse and Gustafsson, 1995). The concentration of L-type channels at the base of dendrites makes them likely candidates for the transduction of distal dendritic activity to the soma where Ca^{2+} influx can in turn modulate gene expression in the nucleus (Westenbroek et al., 1990). Therefore, we examined the effect of L-type Ca^{2+} channel blockers on L-LTP and CRE-mediated transcription. The dihydropyridine, isradipine, (5 μM) markedly inhibited L-LTP after 4–5 hr (Figure 5A). In addition, the increases in CRE-mediated gene expression coincident with L-LTP were significantly attenuated (Figures 5B, 5E, and 5F). Similar results were also obtained with 20 μM nifedipine, another L-type Ca^{2+} channel blocker (data not shown, $n = 3$). The block of the late-component of L-LTP by L-type VGCC inhibitors suggests that L-type Ca^{2+} channel activity may play a critical role in the synthesis of new proteins important for the expression of L-LTP. Furthermore, the requirement for L-type VGCC activity for the concurrent increases in CRE-LacZ expression further strengthens the link between L-LTP and associated increases in CRE-mediated gene expression.

Tetani Which Generate D-LTP or L-LTP Both Elicit Phosphorylation of CREB

Stimulation of CRE-mediated transcription by CREB requires phosphorylation of CREB at Ser-133 (Yamamoto et al., 1988; Gonzalez and Montminy, 1989; Sheng et al., 1991). To examine the relationship between CREB phosphorylation and increases in CRE-mediated gene expression accompanying L-LTP, hippocampal slices were tetanized using stimuli that normally generate D-LTP or L-LTP. CREB phosphorylation was monitored using an antibody specific for CREB phosphorylated at Ser-133 (Ginty et al., 1993, 1994). Tetanization produced a specific increase in phosphorylation of CREB detectable by Western analysis (Figure 6A). Tetani that generated D-LTP or L-LTP both significantly increased phospho-CREB immunoreactivity in area CA1 (Figures 6B–6E). The smaller increase in CREB phosphorylation following the L-LTP stimulus paradigm is probably the result of the longer period of tetanus compared to D-LTP. Phospho-CREB immunoreactivity was significantly reduced 45 min posttetanus. This data demonstrates that LTP in area CA1 of the hippocampus promotes the phosphorylation of CREB at Ser-133. It is noteworthy that D-LTP was associated with CREB phosphorylation but not with increased CRE-LacZ expression. This is consistent with other studies showing that CREB phosphorylation at Ser-133 is not sufficient for CRE-mediated gene expression (Ginty et al., 1994, Sun et al., 1994; Enslen et al., 1994; Schwaninger et al., 1995; Thompson et al., 1995). Phosphorylation of CREB may be necessary but not sufficient for the stimulation of CRE-mediated gene expression in the hippocampus. CRE-mediated transcription is stimulated by kinases other than PKA, including protein kinase C, CaMK IV, $pp90^{rsk}$, and $pp70^{S6k}$ (de Groot et al., 1993; Sun et al., 1994; Enslen et al., 1994; de Groot et al., 1994; Matthews

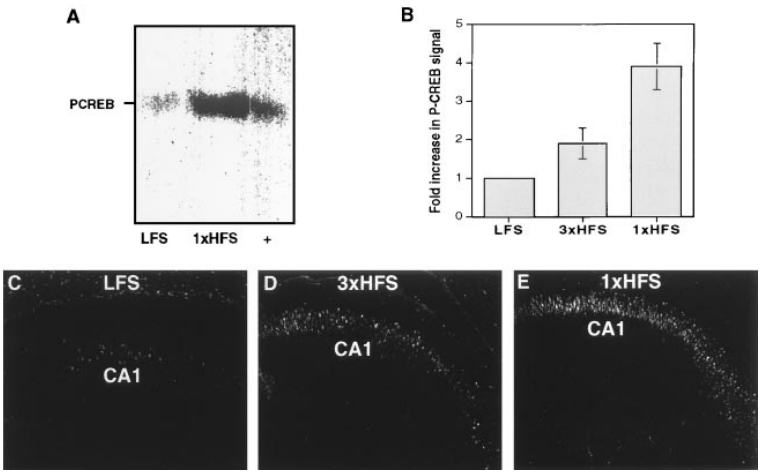

Figure 6. Phosphorylation of CREB by Stimulus Paradigms that Generate D-LTP or L-LTP in Area CA1

(A) A Western blot of phospho-CREB after low frequency stimulation (LFS) and one 100Hz, one s tetanus (1 × HFS). The third lane (+) is a PKA phosphorylated-CREB positive control.

(B) Fold increase in phospho-CREB immunocytochemistry in a slice after LFS, tetanization with a stimulus paradigm (3 × HFS) that generates L-LTP, and tetanized with a stimulus (1 × HFS) that generates D-LTP. Slices were fixed 30 min posttetanus.

(C–E) Representative examples of phospho-CREB immunocytochemistry in a LFS control (C), in a slice that was tetanized with a stimulus paradigm (3 × HFS) that generates L-LTP (D), and in a slice that was tetanized with a stimulus (1 × HFS) that generates D-LTP (E). Images are at 200 × (original) magnification.

et al., 1994; Thompson et al., 1995; Bohm et al., 1995). Thus, increases in CRE-regulated gene expression associated with L-LTP may occur via coordinate activation of multiple signaling pathways and transcription factors.

Discussion

Recent experiments in Drosophila and mice suggest that CREB plays a role in the formation of long-term memory (Yin et al., 1994; Bourtchuladze et al., 1995; Yin et al., 1995). These observations suggest that CRE-regulated gene expression may be involved in the formation of long-term memory in both invertebrates and vertebrates. To explore this hypothesis, we monitored CRE-LacZ expression in response to stimulus paradigms that generate either D-LTP or L-LTP.

CRE-Regulated Gene Expression Is Increased Following LTP

We have demonstrated that L-LTP but not D-LTP is associated with increases in CRE-mediated gene expression. Our data provide the first evidence that the induction of hippocampal long-term synaptic plasticity coincides with increases in CRE-mediated gene expression. The specific induction of CRE-regulated gene expression following L-LTP suggests that CRE-mediated gene expression may be important for the protein synthesis-dependent component of L-LTP. It is interesting to note that L-LTP (in mice) is input specific. Therefore, the possible modulation of synaptic efficacy by CRE-regulated genes must be targeted to activated synapses. This targeting might depend on dendritic protein synthesis, phosphorylation, or proteolysis generated by stimulation of the specific input. CRE-mediated gene expression is also increased by stimulus paradigms that generated long-lasting mossy fiber-LTP and perforant path-LTP. These observations suggest that CRE-regulated protein synthesis may play a general role in the maintenance of synaptic plasticity in the hippocampus.

The Involvement of the cAMP-PKA Pathway

Since activation of PKA is necessary and sufficient for the expression of L-LTP (Matthies and Reymann, 1993; Frey et al., 1993), we examined whether PKA is involved in L-LTP associated increases in CRE-LacZ expression. Treatment of hippocampal slices with the adenylyl cyclase activator, forskolin, generated both L-LTP and associated increases in CRE-mediated gene expression in area CA1. Prior tetanization (that increased CRE-LacZ expression) largely occluded further increases in CRE-LacZ expression by forskolin. Therefore, tetanization and cAMP-mediated increases in CRE-LacZ expression probably share similar mechanisms. Furthermore, inhibition of PKA markedly attenuated both L-LTP and increases in CRE-directed gene expression caused by the L-LTP stimulus paradigm. However, treatment with PKA inhibitors did not prevent D-LTP. This provides additional evidence that the signaling that promotes D-LTP does not stimulate CRE-mediated transcription. Since activation of PKA was sufficient and necessary for increases in CRE-mediated transcription caused by the L-LTP stimulus paradigm, PKA-mediated increases in CRE-regulated gene expression may be required for L-LTP.

Role of Ca^{2+} Influx via L-type Ca^{2+} Channels

D-LTP in area CA1 is dependent on NMDA receptor-mediated Ca^{2+} influx (Collingridge and Bliss, 1995). However, L-LTP in area CA1 can occur independently of NMDA receptor activation. For example, NMDA receptor-independent L-LTP, that requires L-type Ca^{2+} channel activity, can be induced by robust tetanization or blockade of potassium channels (Grover and Teyler, 1990; Aniksztejn and Ben Ari, 1991; Huang and Malenka, 1993; Grover and Teyler, 1994; Hanse and Gustafsson, 1995).

While NMDA receptor inhibitors completely blocked D-LTP in mice, they did not prevent the expression L-LTP or the associated increase in CRE-regulated gene expression. We also explored the role of L-type VGCCs, because they have been implicated in NMDA receptor-independent LTP. L-type Ca^{2+} channels blockers caused a marked reduction in L-LTP, and significantly reduced the associated increases in CRE-mediated gene expression. Collectively, these results suggest that both L-LTP and the increase in CRE-regulated gene expression occur independently of NMDA receptor activation and are at least partially dependent on VGCC activity. This conclusion is reinforced by data that showed that

NMDA did not cause marked increases in CRE-mediated gene expression in cultured neurons (Figure 1E). These data also raise the possibility that L-LTP generated by direct activation of adenylyl cyclase or PKA might also depend on increases in intracellular Ca^{2+}. The inability of L-type Ca^{2+} channel blockers to completely attenuate L-LTP and increases in CRE-regulated gene expression suggests that other sources of Ca^{2+} are involved. Activation of other VGCCs and metabotropic glutamate receptors could contribute to tetanus-induced increases in intracellular Ca^{2+}. Alternatively, Ca^{2+}-independent signaling that increases cAMP via activation of Gs, release of bg, activation PKC, or perhaps direct voltage activation of adenylyl cyclase may be involved (Choi et al., 1993; Taussig and Gilman, 1995; Reddy et al., 1995).

The subcellular localization of L-type Ca^{2+} channels in the somatic membrane and proximal dendrites of hippocampal neurons has led to the proposal that they may serve to couple increases in Ca^{2+} to gene expression (Westenbroek et al., 1990). In support of this hypothesis, the synaptic activation of L-type Ca^{2+} channels was sufficient to induce the expression of CRE-regulated immediate early genes in cultured neurons (Murphy et al., 1991). Furthermore, the induction of CRE-regulated transcription by glutamate in hippocampal neurons required L-type Ca^{2+} channel activity (Bading et al., 1993). In the intact slice, high frequency stimulation of the Schaffer collaterals results in a large influx of Ca^{2+} into the soma and proximal dendrites mediated largely by high-threshold Ca^{2+} channels (Miyakawa et al., 1992; Regehr and Tank, 1992; Christie et al., 1995). This suggests that the activation of VGCCs is of primary importance in the transduction of a tetanus induced Ca^{2+} signal to the nucleus. Our observations that the activation of L-type Ca^{2+} channels is required for both L-LTP and associated increases in CRE-LacZ expression support this hypothesis. However, we cannot exclude the possibility that local dendritic increases in Ca^{2+} modulate somatic intracellular Ca^{2+} or gene expression.

Possible Mechanisms for L-LTP-Associated Increases in CRE-Regulated Gene Expression

There are at least two obvious mechanisms that can account for the dependency of L-LTP-associated increases in CRE-LacZ expression on both the PKA and Ca^{2+} signaling pathways. A Ca^{2+}/CaM-sensitive adenylyl cyclases could be stimulated by VGCC-generated Ca^{2+} influx, thus inducing the activation and nuclear translocation of PKA. The Ca^{2+}/CaM-sensitive adenylyl cyclases, type I (Xia et al., 1991) and type VIII (Cali et al., 1994) are both expressed in the CA1 region of hippocampus. Alternatively, a Ca^{2+} stimulated protein kinase or phosphatase that modulates CRE-regulated transcription may be potentiated by PKA-mediated enhancement of VGCC Ca^{2+} influx. The requirement for both the PKA and Ca^{2+} signaling pathways may also reflect synergistic increases in CRE-mediated gene expression. For example, activation of the type I adenylyl cyclase by both Gs and Ca^{2+} synergistically increased CRE-mediated gene expression (Impey et al., 1994). On the other hand, in PC12 cells and hippocampal neurons, the pairing of activators of PKA with agents that increase intracellular Ca^{2+} synergistically increased CRE-regulated gene expression in the absence of adenylyl cyclase activation (Van-Nguyen et al., 1990; Sheng et al., 1990; S.I. and D.R.S., unpublished data). However, the mechanisms that underlie this synergistic stimulation of CRE-regulated gene expression in neuronal cells remain unclear.

Members of the CREB/ATF family can promote CRE-regulated gene expression via Ca^{2+} or cAMP and are likely to mediate induction of CRE-LacZ caused by the L-LTP stimulus paradigm. However, just as the signaling pathways that converge on the CRE have multiplied, the transcription factors that can mediate transactivation have also diversified to include CREB/ATF heterodimers with other b-zip transcription factors (Hai and Curran, 1991; Vallejo et al., 1993; Chatton et al., 1994; De-Cesare et al., 1995). Nevertheless, CREB was phosphorylated at Ser-133, following the induction of LTP, and it may contribute to L-LTP-associated increases in CRE-mediated gene expression.

Although we provide evidence that CRE-regulated gene expression may be causally involved in the maintenance of LTP, the identity of the newly transcribed genes crucial for L-LTP is not yet known. Several of the CRE-regulated immediate early genes induced by LTP in the dentate gyrus are also transcription factors. Therefore, it is likely that at least some of the genes involved in the maintenance of L-LTP are delayed response genes induced by L-LTP-induced immediate early genes. Although the pattern of gene expression induced by LTP is almost certainly complex, it is noteworthy that many genes involved in neurotransmission are thought to be regulated via the CRE pathway.

In summary, this is the first report showing that CRE-mediated gene expression is induced by stimuli that generate L-LTP in the hippocampus. Furthermore, these results suggest that CRE-mediated gene expression is involved in the maintenance of L-LTP. These data are especially interesting in the context of recent work suggesting that CREB-regulated transcription may be important for long-term memory in Drosophila and mice (Bourtchuladze, 1994; Yin et al., 1994 and 1995), and support the hypothesis that the regulation of CRE-mediated transcription by the CREB/ATF family of transcription factors is fundamentally involved in the formation of long-term memory in both invertebrates and vertebrates.

Experimental Procedures

Creation of the CRE-LacZ Transgenic Mouse

The CRE-LacZ construct used for the generation of transgenic mice contained six tandem CREs upstream from a minimal RSV promoter that drives the expression of β-galactosidase (Meinkoth et al., 1990). The mice were generated using C57BL6/SJL F2 blastocysts for microinjection. Founders were bred to C57BL6 mice and animals were maintained and bred under standard conditions. PCR was used to screen for genotype.

Neuron Culture

Primary hippocampal neurons were cultured essentially as described in Impey et al., 1994, and were maintained in NeuroBasal (GIBCO BRL) with B27 supplement (GIBCO BRL). Briefly, dissected brain areas were minced in Hank's balanced salt solution containing 10 mM $MgCl_2$ (HBSS+Mg). The tissue was then incubated in HBSS+Mg containing 1 mg/ml activated papain (Worthington Biochemicals) at 37°C for 30 min. The tissue was then rinsed with

HBSS+Mg and dissociated via gentle passage through a 2 ml serological pipette. Cells were plated at $5x10^5$ cells per well in 12 well plates.

β-Galactosidase Assay

Neurons were incubated in HBSS with effectors for 2 hr. In the case of NMDA treatment, the HBSS lacked extracellular Mg^{2+} and was supplemented with 1 mM glycine. The media was changed to conditioned neuronal growth medium and the neurons were incubated at 37°C for 4 hr. The neurons were lysed with 0.2% Triton X-100 (Sigma),100 mM sodium phosphate, and 1 mM DTT at pH 7.8. β-galactosidase was assayed using the Galacton™ chemiluminescent substrate (AMPGD) with the Galacto-Light system (Tropix). The assay was carried out essentially as described in Matthews et al., 1994, except that 100 mM D-galactose was added to the substrate buffer to reduce lysosomal β-galactosidase background. Chemiluminescence was measured with a Berthold luminometer (Tropix). Relative β-galactosidase activity was normalized to protein by the bicinchoninic acid method (Pierce Chemical).

Agonist Treatment of Perfused Hippocampal Slices

Transverse 400 μm hippocampal slices were continuously perfused with oxygenated Krebs-bicarbonate buffer (120 mM NaCl, 3.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25.6 mM $NaHCO_3$, and 10 mM glucose, and aerated with 95% O_2, 5% CO_2 at 34°C for 1–2 hr before addition of forskolin (Calbiochem), or 1,9-dideoxy-forskolin (Calbiochem). Forskolin was added to the perfusate for 1 hr. A tungsten bipolar stimulating electrode (100 μm diameter) and low resistance glass microelectrodes (1–2 μm tip) filled with 3 mM NaCl were used for recording. Pulses consisting of 0.3 ms square waves were delivered by a Grass S11 Stimulator. Stimulus intensity was adjusted to produce 33% of the maximal EPSP amplitude.

Electrophysiology

For LTP in area CA1, transverse 400 μm hippocampal slices were continuously perfused with oxygenated Krebs-bicarbonate buffer at 34°C for 1–2 hr before electrophysiological studies were conducted. Schaffer collateral afferent fibers in the stratum radiatum were stimulated and field EPSP responses were elicited at 0.02 Hz (LFS). A tungsten bipolar stimulating electrode (100 μm diameter) and low resistance glass microelectrodes (1–2 μm tip) filled with 3 M NaCl were used for recording. Pulses consisting of 0.3 ms square waves were delivered by a Grass S11 Stimulator. Potentiated slices were perfused for at least 4 hr to allow the development of CRE-LacZ expression. L-LTP was produced with three, 100 Hz, one s tetani presented at 5 min intervals (pulse length 0.3 ms). Two pathway experiments indicated that L-LTP was input specific (data not shown). D-LTP was produced with one, 100 Hz, one s tetanus (pulse duration 0.1 ms). H89 (Biomol) was perfused for the length of the experiment. KT5720 (LC Laboratories), D,L APV (Sigma), Isradipine (RBI), and MK801 (RBI) were perfused from 30 min pretetanus, to 1 hr posttetanus. Stimulus intensity was adjusted to produce 33% of maximal EPSP amplitude.

Immunocytochemistry

Hippocampal slices were fixed and cryoprotected in 2% paraformaldehyde and 30% sucrose in phosphate buffered saline (PBS) and were sectioned at 50 μm intervals. Immunocytochemistry for β-galactosidase was performed using standard procedures with a rabbit anti-β-galactosidase antibody at 1:1000 dilution (Cappel) and Texas-Red goat-anti-rabbit IgG at 2 μg/ml (Jackson Immunochemicals). Immunocytochemistry for β-galactosidase and the neuronal marker MAP-2 (Santa Cruz) demonstrated that the majority of LacZ expressing cells were neurons. For immunocytochemistry of phospho-CREB, slices were fixed and cryoprotected in ice cold PBS containing 4% paraformaldehyde, 30% sucrose, and 100 mM NaF for 30 min following the last tetanus. A triple label procedure was used: phospho-CREB antibody was used at 1:1000 dilution (Ginty et al., 1993), biotin labeled goat-anti-rabbit IgG was used at 10 μg/ml (Jackson Immunochemicals), and LRSC labeled streptavidin was used at 5 μg/ml (Jackson Immunochemicals). Images were captured on a Bio-Rad MRC-600 laser scanning confocal microscope.

Quantification of Immunocytochemistry

Quantification was carried out on a DTPpro pentium computer with Metamorph software. Briefly, integrated pixel intensity was measured in the desired region. A constant pixel area was used for three independent measurements that were averaged. The averaged integrated pixel intensity for groups of slices were statistically analysed. The unpaired two tailed Student's t-Test was used to test for significance. Imaging and quantitation was conducted blind.

Western Blot Analysis of CREB Phosphorylation

Treated amd stimulated as described above were 400 μm mini-slices containing only area CA1 and the proximal area CA3. For thirty min following the last tetanus, the CA1 layer was dissected and frozen in an ethanol dry ice bath. Upon thawing, the tissue (c. 50 slices) was washed with ice cold PBS and sonicated in lysis buffer (50 mM Tris [pH7.4], 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM NaVO4, 1 mM PMSF, 25 μg/ml leupeptin, and 25 μg/ml aprotinin). The lysate was spun 20 min at 14,000 g. Sample buffer (40 mM Tris [pH6.9], 2 mM EGTA, 10% glycerol, 1% dithiothreitol, 1% SDS, 0.04% bromophenol blue) was added to the supernatant that was then boiled for 10 min. Cell extracts were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked with 4% cold fish gelatin in PBST and then incubated with 0.14 μg/ml anti-phospho-CREB (Ginty et al., 1993). Blots were developed using enhanced chemiluminscence (Amersham).

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